Different Pattern of Collagen Cross-Links in Two Sclerotic Skin Diseases: Lipodermatosclerosis and Circumscribed Scleroderma

J. Brinckmann, C.M. Neess,* Y. Gaber, H. Sobhi,* H. Notbohm,† N. Hunzelmann,* P.P. Fietzek,† P.K. Müller,† J. Risteli,‡ R. Gebker, and K. Scharffetter-Kochanek*

Department of Dermatology, Medical University of Lübeck, Germany; †Institute for Medical Molecular Biology, Medical University of Lübeck, Germany; *Department of Dermatology, University Cologne, Germany; ‡Department of Clinical Chemistry, University of Oulu, Finland

Changes in the process of cross-linking of collagen molecules are associated with defects in the biomechanical stability of the extracellular matrix. Fibrosis of skin is characterized by an increase in pyridinolines, which are hydroxylysine aldehyde derived cross-links usually absent in healthy skin. In this study, we analyzed cross-links in lipodermatosclerosis and localized scleroderma to address the question whether all the mature cross-links currently characterized are increased in fibrosis in addition to the increase in pyridinolines. As psoralen plus ultraviolet A treatment leads to clinical improvement of fibrotic plaques in localized scleroderma we analyzed the cross-link content in lesional skin after bath psoralen plus ultraviolet A therapy. In skin from patients with localized scleroderma an increase in the total number of mature cross-links was found to be due to an increase in both pyridinolines and dehydro-histidinohydroxymerodesmosine. The concentration of histidinohydroxylysinonorleucine was unchanged. By contrast, the total number of mature cross-links was decreased in lipodermatosclerosis. This decrease was caused by a decrease of lysine aldehyde derived cross-links (dehydro-histidinohydroxymerodesmosine and histidinohydroxylysinonorleucine), whereas the concentration of pyridinolines increased. A decrease in the content of pyridinolines after bath psoralen plus ultraviolet A treatment was found in six out of nine patients with localized scleroderma, which might reflect a remodeling of the extracellular matrix. Our data provide evidence that sclerosis of skin is associated with either an increase in the number of cross-links per molecule of collagen or a change in the molecular nature of the cross-links formed.

Key words: cross-links/PUVA/pyridinolines/sclerosis/UV irradiation. J Invest Dermatol 117:269–273, 2001

An appropriate stabilization of collagen molecules by intermolecular collagen cross-links is crucial for the proper biomechanical function of tissues and organs. The Ehlers–Danlos syndrome type VI, which is due to a mutation in the gene of lysylhydroxylase, is accompanied by changes in the mode of cross-linking leading to hyperflexibility of joints and an increased fragility of blood vessels (Hyland *et al*, 1992; Açil *et al*, 1995). Collagen cross-links consist of a group of mature compounds, which are endproducts of a complex process initiated by the lysyl-oxidase-dependent oxidative deamination of lysine (lys) or hydroxylysine (hyl) residues in telopeptides forming reactive aldehydes. Subsequent condensation results in the formation of difunctional intermediate products and trifunctional or tetrafunctional cross-links. Structurally, the cross-links consist of two groups comprising (i) compounds that are derived from a lys residue in the telopeptides of the collagen molecule [dehydro-histidinohydroxymerodesmosine (Δ-HHMD), histidinohydroxylysinonorleucine (HHL)] and (ii) compounds that require a hyl residue in the telopeptide [pyridinolines: hydroxylysylpyridinoline (HP), lysylpyridinoline (LP); pyrrole]. As the lys residue in the telopeptide of collagen in skin is probably not hydroxylated, HHL and Δ-HHMD are generally found, whereas pyridinolines (HP and LP) occur only in traces (Barnes *et al*, 1971; Yamauchi *et al*, 1987). In contrast, the lys residues in the telopeptides of skeletal tissue are hydroxylated, resulting in the generation of HP, LP, and pyrrole as mature cross-links (Eyre *et al*, 1984; Knott and Bailey, 1998). Recent data showed that sclerosis of skin [lipodermatosclerosis (LDS), keloid] or organ fibrosis (lung, liver) is accompanied by an increase in the hyl-derived cross-links HP and LP. This is due to an increased hydroxylation of the collagen molecule in both the helical part and the telopeptide, which in turn determines the type of cross-links (Reiser *et al*, 1992; Ricard-Blum *et al*, 1993; Brinckmann *et al*, 1996; Uzawa *et al*, 1998). Furthermore, an increase in HHL content was recently reported for sclerotic skin of systemic sclerosis and for skin after radiotherapy (Ishikawa *et al*, 1998; Sassi *et al*, in press). It is open to debate, however, whether the increase in hydroxylysine aldehyde (hyl-ald) derived cross-links is due to an isolated overhydroxylation of lys residues or to an increase in the total number of all mature cross-links caused by a stimulated expression of lysyl oxidase. In this

Manuscript received November 14, 2000; revised March 7, 2001; accepted for publication April 3, 2001.

Reprint requests to: Dr. med. Jürgen Brinckmann, Department of Dermatology, Medical University of Lübeck, Ratzeburger Allee 160, 23552 Lübeck, Germany. Email: Brinckmann@molbio.mu-luebeck.de

Abbreviations: Δ-HHMD, dehydro-histidinohydroxymerodesmosine; HHL, histidinohydroxylysinonorleucine; HP, hydroxylysylpyridinoline; hyl, hydroxylysine; hyl-ald, hydroxylysine aldehyde; hyp, hydroxyproline; LDS, lipodermatosclerosis; LP, lysylpyridinoline; lys, lysine; lys-ald, lysine aldehyde.

study, we compared the pattern of mature collagen cross-links (HP, LP, HHL, Δ-HHMD) in localized scleroderma and in LDS. As 8-methoxypsoralen (8-MOP) plus ultraviolet A (PUVA) therapy is an effective treatment modality for localized scleroderma (Yamauchi *et al*, 1991; Kerscher *et al*, 1994; Scharffetter-Kochanek *et al*, 1995; Stege *et al*, 1997), we set out to analyze the influence of bath-PUVA treatment on the concentration of cross-links *in vitro* and in localized scleroderma *in vivo*.

MATERIALS AND METHODS

Human skin samples Skin samples were obtained from patients with LDS undergoing reconstructive surgery of sclerotic areas and from patients with localized scleroderma before and directly after the last bath-PUVA treatment of a total of 36 treatments (LDS, $n = 8$; localized scleroderma, $n = 9$). Age- and site-matched controls were obtained from normal skin of patients undergoing plastic surgery ($n = 9$). All biopsies from patients (LDS and localized scleroderma) and from controls were performed after the written consent of the donors had been obtained.

Bath-PUVA treatment Nine patients with histologically confirmed localized scleroderma were treated with bath-PUVA for 12 wk (total of 36 treatments). During the first 6 wk they were treated four times a week (24 treatments), and treatments were subsequently reduced to twice a week. Before UVA exposure the minimal phototoxic dose was determined. The initial UVA dose was started with 0.2–0.5 J per cm^2 corresponding to 30% of the previously determined minimal phototoxic dose. For irradiation we used a stand-up cubicle emitting UVA isodoses at every given anatomic side (Medisun, Schulze & Boehm, Cologne, Germany). The concentration of 8-MOP was 0.5 mg per liter, the water temperature was 37°C, and the patients bathed for 20 min. Ultrasonography (20 DUB, 20 MHz tpm; Lüneburg, Germany) of lesional and nonlesional skin at baseline and after treatment was performed. No serious side-effects such as erythema, pruritus, or severe burning was observed by any patients.

Pyridinoline analysis (HP, LP) Skin samples were acid hydrolyzed (6 N HCl, 110°C, 24 h). Fluorescent collagen cross-links HP and LP were measured in acid hydrolysates by high performance liquid chromatography after purification by adsorption chromatography (fibrous cellulose powder column, CF-1; Whatman, Springfield Mill, Maidstone, U.K.) (Brinckmann *et al*, 1996). Chromatography was carried out on an Inertsil 5 mm column from VDS Optilab using a fluorescence detector RF 1002 from Gynkothek (Gynkothek, Germering, Munich, Germany). Samples were eluted with a gradient from 5% to 65% acetonitrile in 0.1% heptafluorobutyric acid over 25 min at room temperature. Quantitation of HP and LP was based on calibration with suitable standards prepared from gelatine. Collagen quantity was calculated from the content of hydroxyproline (hyp) in acid hydrolysates assuming a content of 14 mg hyp in 100 mg collagen. Hyp was measured in a color assay. After acid hydrolysis, dried aliquots were oxidized with chloramine T and reacted with dimethylaminobenzaldehyde. The color value was measured at 550 nm and normalized to hyp standard solutions (Bergman and Loxley, 1963).

Content of lysyl-oxidase-dependent collagen cross-links (HHL, Δ-HHMD) Acid hydrolysates of borohydride-reduced and nonreduced samples were initially separated on CF-1 cellulose columns to remove the bulk of non-cross-linked amino acids. Dried eluates were redissolved in Na-S buffer and analyzed on the amino acid analyzer (Beckmann, Germany) using a two buffer gradient system and post column ninhydrin derivatization. The column was eluted for 20 min with Na-F buffer and for 40 min with Na-E buffer at 90°C (Beckmann, Germany). Retention times of individual cross-links were established with authentic cross-link compounds. Quantification was based on ninhydrin-generated leucine equivalence factors using 1.97 for HHL. In analogy to the factors published for HP and desmosine the factor for Δ-HHMD was arbitrarily assumed to be 3.4 (Sims *et al*, 2000).

Amino acid analysis The degree of lysyl hydroxylation was analyzed in hydrolyzed full-thickness biopsies. Lyophilized samples were redissolved in sample buffer for amino acid analysis (Na-S) and separated with an amino acid analyzer using a step gradient of 7.3 ml buffer Na-F and 3.2 ml Na-D (Beckmann, Germany). The degree of lysyl hydroxylation was expressed as the ratio of hyl to hyp.

***In vitro* UVA irradiation** HP-containing collagen was isolated by repeated pepsin digestion (0.1 mg pepsin per mg dry weight of skin, for 24 h at 4°C; five times) of skin with LDS and salt precipitated (4.0 M NaCl). Skin of LDS was pulverized under liquid nitrogen in a hammer mill. Aliquots of redissolved collagens (0.2 M NaCl, pH 7.4) or of pulverized skin were irradiated in the presence and absence of 8-MOP (80 ng per ml) with different doses of UVA (UVASUN 3000, Mutzhaas, Munich, Germany). The irradiation was performed once (9 J per cm^2) or repetitively (3×9 J per cm^2). After irradiation of the PUVA sample, 8-MOP was removed from the collagen solution by dialysis and from the skin by washing. Subsequently, an aliquot of a freshly prepared 8-MOP solution was added prior to the next irradiation. All experiments were performed in triplicate.

Radioimmunoassays Carboxyterminal telopeptide of collagen I was measured in 100 μl aliquots of serum in duplicate using equilibrium radioimmunoassays for the human antigen (Orion Diagnostica, Oulunsalo, Finland). Briefly, after incubation of samples with the radiolabeled antigen and rabbit antiserum for 24 h at 37°C, the second antibody was added for 30 min at 4°C. The precipitate was collected by centrifugation and counted in a 1272 Clinigamma counter (Wallac, Turku, Finland), which calculates the standard curve and automatically gives the result on the basis of this. The intraassay and interassay coefficients of variation were about 5% for both assays at the concentration found in this study.

Electrophoretic separation Aliquots of solubilized collagens were lyophilized and redissolved in sodium dodecyl sulfate (SDS) sample buffer in a concentration of 1 mg per ml. Each sample was heated to 95°C for 2 min and quenched on ice prior to sample loading. Separation of collagen chains on polyacrylamide gels was carried out in the presence or absence of 2-mercaptoethanol in sample buffer and also under conditions with delayed reduction. Gels were stained with Coomassie blue and relative amounts of the different collagen chains were measured by densitometric scanning using a video scanner for whole band analysis calibrated by a photographic step tablet (Computer & Vision, Lübeck, Germany; Kodak, Rochester, NY).

Circular dichroism and transition profiles Circular dichroism spectra were recorded in a Jasco J-715 A spectropolarimeter, equipped with a temperature-controlled quartz cuvette of 1 cm path length. The molar ellipticity was calculated on the basis of a mean residue molar mass of 96 g per mol. The degree of 100% conversion corresponds to the ellipticity at 45°C, characteristic for totally denatured collagen. Thermal transition curves were recorded at a fixed wavelength (221 nm) by raising the temperature linearly at a rate of 30°C per h, using a Gilford temperature programmer.

Statistical analysis Statistical analysis was based on the *U* test, according to Wilcoxon, Mann–Whitney, and on the Wilcoxon matched pairs signed rank test.

RESULTS

Hyl-ald derived cross-links and the degree of lysyl hydroxylation increase in lesional skin of localized scleroderma An increase in the content of pyridinolines occurred in lesional skin of localized scleroderma compared with healthy controls (localized scleroderma, HP 31 ± 18, LP 2.2 ± 1.1 mmol per mol; control, HP 5 ± 3.2, LP 0.4 ± 0.8 mmol per mol; $p < 0.005$). In LDS, both pyridinolines showed an increase, which was not as pronounced as reported earlier (Brinckmann *et al*, 1996). There is a distinctive difference between the two pathologic conditions, however: in LDS, the increase of hyl-ald derived cross-links was mainly due to a proportionally higher formation of HP, whereas the formation of the two pyridinolines was found to be stimulated to a similar extent in localized scleroderma (**Fig 1*a*, *b***). The increase in pyridinolines in localized scleroderma was accompanied by a slight but significant elevation of the degree of lysyl hydroxylation in total skin hydrolysates expressed as hyl/hyp (localized scleroderma, 0.061 ± 0.006; control, 0.048 ± 0.010; $p < 0.01$). The increase in pyridinolines in the skin of localized scleroderma was not accompanied by an increase in the serum concentration of the carboxyterminal telopeptide of collagen I (data not shown).

Lys-ald derived cross-links increase in localized scleroderma and decrease in LDS In order to address the question whether the increase in the concentration of pyridinolines is paralleled by a

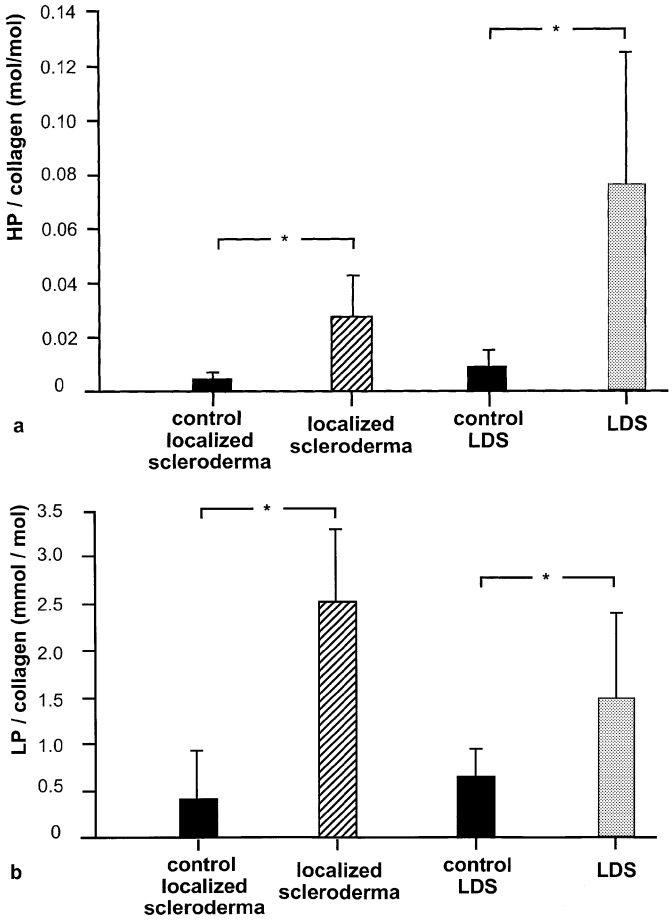

Figure 1. Increased content of pyridinolines in skin of localized scleroderma and LDS. (*a*) HP, (*b*) LP. Fluorescent collagen cross-links HP and LP were measured in acid hydrolysates. Each bar corresponds to the mean ± SD, *p < 0.005.

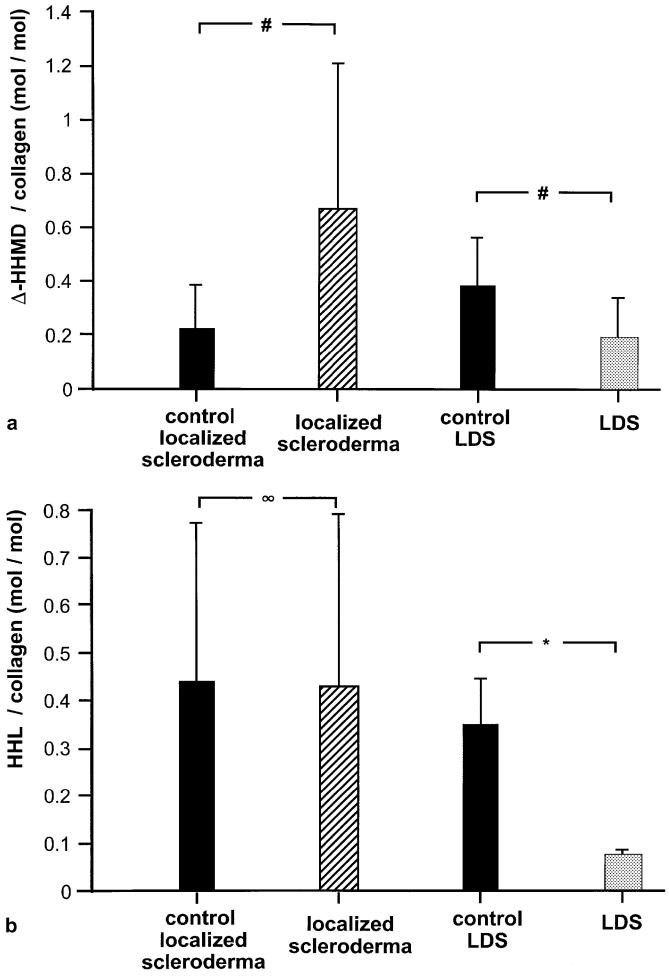

Figure 2. The pattern and content of lys-derived cross-links distinctly differ in skin of localized scleroderma and LDS. (*a*) Δ-HHMD, (*b*) HHL. Acid hydrolysates of borohydride-reduced and nonreduced samples were analyzed on the amino acid analyzer using a two buffer gradient system and post column ninhydrin derivatization. Each bar corresponds to the mean ± SD; #p < 0.05, ∞p > 0.05, *p < 0.005.

general increase in all mature cross-links, we analyzed the concentration of mature lys-ald derived compounds in localized scleroderma and LDS. Interestingly, an increase in the concentration of Δ-HHMD was observed in skin of localized scleroderma, whereas this concentration was decreased in LDS (**Fig 2*a***). The concentration of HHL was virtually unchanged in localized scleroderma, whereas in LDS a marked decrease in HHL was observed (**Fig 2*b***). The concentration of both Δ-HHMD and HHL in the skin of localized scleroderma was highly variable. This variation may reflect differences in disease stage, age, and site of biopsy. It should also be noted that the concentration of cross-links in the two control pools used as reference for either localized scleroderma or LDS varied to some extent, which similarly may be attributed to differences in age and site of biopsy. Taken together, total mature cross-links (lys-ald and hyl-ald derived cross-links) increase from 0.67 ± 0.46 mol per mol in controls to 1.13 ± 0.53 mol per mol in localized scleroderma ($p < 0.05$), whereas a decrease was observed in LDS (control, 0.75 ± 0.23 mol per mol; LDS, 0.33 ± 0.16 mol per mol; $p < 0.005$).

***In vitro* UVA and PUVA treatment degraded HP in intact collagen molecules** In order to study photodegradation of pyridinolines due to UVA or PUVA treatment, we performed an *in vitro* irradiation of pepsin-solubilized collagen with a known predetermined concentration of pyridinolines and of powdered skin of LDS. The UVA irradiation of intact collagen molecules containing HP resulted in a decrease in HP content. Repetitive irradiation (three times) resulted in enhanced photodegradation of HP, whereas PUVA treatment of the collagen solution did not result in a further increase in photodegradation of HP compared to the UVA treatment. Furthermore, UVA treatment of pulverized skin resulted in a decrease in HP content. PUVA treatment of the skin *in vitro* showed a slight increase in the degradation of HP after repetitive irradiation compared to the UVA treatment (**Table I**). Following UVA and PUVA treatment of collagen solutions no structural changes and no fragmentation of collagen were observed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (**Fig 3**). The relative proportions of γ-, β- or α-collagen chains and of collagen III to collagen I did not vary (data not shown). Furthermore, thermal transition curves of irradiated and mock-irradiated collagen solutions showed no substantial differences in the melting profile of collagen molecules (data not shown).

Bath-PUVA treatment of lesional skin in localized scleroderma resulted in a decrease in HP content in six out of nine patients In six out of nine patients with localized scleroderma, a decrease in HP content was observed after bath-PUVA, the extent of which varied between patients from 23% to 73% ($p < 0.05$). One patient did not show any change, but in two patients increases in HP content of 30% and 40% were observed (**Fig 4**). A decrease in LP content after bath-PUVA therapy was also observed ($p < 0.05$). The degree of lysyl hydroxylation was not altered by bath-PUVA therapy (hyl/hyp before treatment 0.061 ± 0.006, after treatment

Table I. UVA and PUVA treatment *in vitro* degraded HP in intact collagen molecules and pulverized skin[a]

	Mock irradiation	UVA 9 J per cm^2	PUVA 9 J per cm^2	UVA 3×9 J per cm^2	PUVA 3×9 J per cm^2
Collagen solution	0.024	0.009 ± 0.0017	0.011 ± 0	0.006 ± 0.0006	0.006 ± 0.0006
Pulverized skin	0.072	0.019 ± 0.0040	0.021 ± 0.0010	0.020 ± 0.0045	0.014 ± 0.0006

[a]Mean ± SD, $n = 3$.

0.063 ± 0.005; $p > 0.05$). Ultrasonographic measurements of lesional skin performed at baseline and after treatment showed a reduction in sonographic thickness (diameter of dermis before treatment 2.4 ± 0.5 mm, after treatment 2.1 ± 0.5 mm; $p < 0.05$). The HP content in lesional skin before and after treatment did not correlate with skin thickness. Clinically, the PUVA treatment resulted in an improvement of sclerosis in all patients.

DISCUSSION

The pattern and formation of lys-ald and hyl-ald derived collagen cross-links are thought to be determined by physiologic requirements and not collagen types and their proportion in tissues. Hyl-ald derived cross-links are found in mineralized tissues such as bone and dentine as well as in cartilage, whereas they are virtually absent in skin and cornea (Reiser *et al*, 1992; Yamauchi *et al*, 1996; Bailey *et al*, 1998). The initial step in collagen cross-link formation, which apparently limits the total amount of enzymatic cross-links between collagen molecules, is the oxidative deamination of certain lys and hyl residues located in the telopeptide region of collagen molecules. It has been shown in animal experiments that the decline in the concentration of collagen cross-links after inhibition of lysyl oxidase by addition of β-aminoproprionitrile parallels the decrease in biomechanical strength of the tissue studied (Oxlund *et al*, 1995; Bruel *et al*, 1998). An increase in cross-links of the pyridinoline type was observed in sclerotic conditions such as nephrosclerosis, lung fibrosis or LDS, and hypertrophic scars (Moriguchi and Fujimoto, 1979; Last *et al*, 1990; Brinckmann *et al*, 1996; Di Donato *et al*, 1997). It is open to debate, however, whether this increase is due to a general increase in total number of cross-links or to an exclusive increase in HP. In our study, the total number of analyzed cross-links per collagen molecule showed an increase in localized scleroderma. The pattern of cross-links was characterized by an increase in pyridinolines and Δ-HHMD, whereas the concentration of HHL was not altered, an observation that was previously reported for keloids (Uzawa *et al*, 1998). The increase in the concentration of hyl-derived cross-links was not accompanied by an increase in the serum concentration of carboxyterminal telopeptide of collagen I. This may be due to the overall small area of skin involved in our patients (less than 15%) and to the fact that the absolute levels of pyridinolines in localized scleroderma were low compared to skeletal tissues (Hunzelmann *et al*, 1998). In lesional skin of LDS a decrease in the content of cross-links analyzed was observed, which was due to a marked decrease in HHL and Δ-HHMD. The increase in the concentration of pyridinolines in LDS was not as marked as reported earlier probably due to different stages of disease (Brinckmann *et al*, 1996).

The reason why different cross-link patterns are formed in localized scleroderma and LDS is not clear, although it is conceivable that stimulated expression of lysyl oxidase in localized scleroderma resulted in an overall increase in the concentration of cross-links per collagen molecule (Chanoki *et al*, 1995). The higher increase in hyl-ald derived cross-links (+615%) than in lys-ald derived cross-links (+164%) argues for an additional stimulation of the expression of lysyl hydroxylase in localized scleroderma. Our observation of a slight but significant elevation of the degree of lys hydroxylation in whole skin samples is in line with this notion.

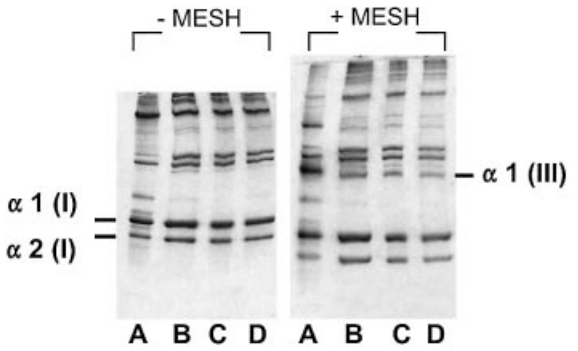

Figure 3. No fragmentation of soluble collagen following UVA or PUVA treatment. HP-containing collagen was isolated by repeated pepsin digestion (0.1 mg pepsin per mg dry weight of skin, for 24 h at 4°C; five times) of skin with LDS. After three treatments with 9 J per cm^2 UVA in the presence or absence of 8-MOP collagens were lyophilized and separated by SDS-PAGE. Electrophoresis was performed in the absence (–MESH) or presence (+MESH) of mercaptoethanol (*A*, standard of pepsin solubilized bovine skin collagen; *B*, mock-irradiated control; *C*, PUVA; *D*, UVA).

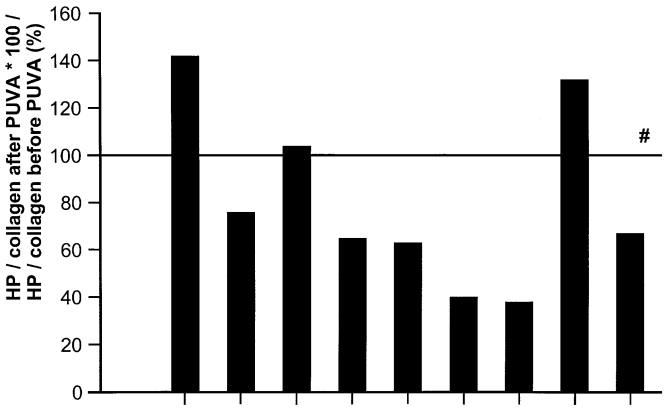

Figure 4. Bath-PUVA treatment of lesional skin in localized scleroderma resulted in a decrease in HP content in six out of nine patients. HP content in skin of localized scleroderma after PUVA therapy is expressed as a percentage of HP content before PUVA therapy. Each bar represents the HP content of a patient; #$p < 0.05$.

By contrast, the altered cross-link pattern in LDS seems to be due to an isolated overhydroxylation of lysyl residues of the collagen molecules leading to a decrease in lys-ald derived cross-links and a sole increase in pyridinolines (Brinckmann *et al*, 1999). Furthermore, it is conceivable that stimulated expression of both lysyl oxidase and lysyl hydroxylase in a first step leads to an increase in lys-ald and hyl-ald derived cross-links. In a second step lys-ald derived cross-links may be cleaved in preference to pyridinolines by collagenase, the expression of which has been found to be enhanced in LDS (Vater *et al*, 1974; Herouy *et al*, 1998).

Several studies have shown clinical improvement following bath-PUVA treatment in localized scleroderma, which was explained by an increase in synthesis and activation of matrix metalloproteinases with proteolytic specificity for interstitional collagens (Scharffetter *et al*, 1991; Petersen *et al*, 1992; Herrmann *et al*, 1998). We found in six out of nine patients a decrease in the content of pyridinolines after bath-PUVA, reflecting a remodeling of the extracellular matrix probably induced by an altered cytokine profile. We did not observe a decrease in the sclerosis-specific cross-link HP in three patients with localized scleroderma, which may be due to different concentrations of dehydro-dihydroxylysinonorleucine DHLNL at the time of biopsy. In addition to the effect of remodeling of sclerotic skin it is conceivable that a direct photodegradation may contribute to the removal of pyridinolines (Sakura *et al*, 1982; Meddah *et al*, 2000). Our *in vitro* irradiation of solubilized collagen and pulverized skin clearly showed that HP bound to intact collagen molecules was effectively degraded by UVA. PUVA treatment *in vitro* apparently enhanced the UVA-induced photodegradation of HP only when repetitively performed on pulverized skin. The generation of reactive oxygen species upon UVA irradiation of 8-MOP and chromophores present in skin may account for this effect (Scharffetter-Kochanek *et al*, 2000). The irradiation of collagen with UVA in the presence or absence of 8-MOP did not lead to any structural changes or to fragmentation of collagen.

In summary, our study provides first evidence that sclerosis of skin is accompanied by a change in the quantitative and/or qualitative formation of collagen cross-links. The role that lys-ald and hyl-ald derived collagen cross-links have in changing the physicomechanical properties of skin tissue is open to debate.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 367-A7), the Deutsche Gesellschaft für Phlebologie, and the Bundesministerium für Bildung und Forschung (BMBF 07U5B55B/1). We thank Mrs Fischer and Mrs Alexander for their excellent technical assistance.

REFERENCES

Açil Y, Vetter U, Brenner R, Müller PK, Brinckmann J: Diagnosis of Ehlers–Danlos syndrome type VI. Cross-link pattern in tissue and urine sample as a diagnostic marker. *J Am Acad Dermatol* 33:522–524, 1995

Bailey AJ, Paul RG, Knott L: Mechanisms of maturation and ageing of collagen. *Mech Ageing Dev* 106:1–56, 1998

Barnes MJ, Constable BJ, Morton LF, Kodicek E: Hydroxylysine in the N-terminal regions of the 1′- and 2′-chains of various collagens. *Biochem J* 125:433–437, 1971

Bergman I, Loxley R: Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Anal Chem* 35:1961–1965, 1963

Brinckmann J, Açil Y, Tronnier M, *et al*: Altered x-ray diffraction pattern is accompanied by a change in the mode of cross-link formation in lipodermatosclerosis. *J Invest Dermatol* 107:589–592, 1996

Brinckmann J, Notbohm H, Fietzek P, Tronnier M, Schmeller W, Bätge B, Müller PK: Over-hydroxylation of lysyl residues is the initial step for altered collagen cross-links and fibril architecture in fibrotic skin. *J Invest Dermatol* 113:617–621, 1999

Bruel A, Ortoft G, Oxlund H: Inhibition of cross-links in collagen is associated with reduced stiffness of the aorta in young rats. *Atherosclerosis* 140:135–145, 1998

Chanoki M, Ishii M, Kobayashi H, Fushida H, Yashiro N, Hamada T, Ooshima A: Increased expression of lysyl oxidase in skin with scleroderma. *Br J Dermatol* 133:710–715, 1995

Di Donato A, Ghiggeri GM, Di Duca M, Jivotenko E, Acinni R, Campolo J, Ginervi F, Gusmano R: Lysyl oxidase expression and collagen cross-linking during chronic adriamycin nephropathy. *Nephron* 76:192–200, 1997

Eyre DR, Paz MA, Gallop PM: Cross-linking in collagen and elastin. *Annu Rev Biochem* 537:717–748, 1984

Herouy Y, May AE, Pornschlegel G, *et al*: Lipodermatosclerosis is characterized by elevated expression and activation of matrix metalloproteinases: implications for venous ulcer formation. *J Invest Dermatol* 111:822–827, 1998

Herrmann G, Brenneisen P, Wlaschek M, *et al*: Psoralen photoactivation promotes morphological and functional changes in fibroblast *in vitro* reminiscent of cellular senescence. *Cell Sci* 111:759–767, 1998

Hunzelmann N, Risteli J, Risteli L, Sacher C, Vancheeswaran R, Black C, Krieg T: Circulating type I collagen degradation products: a new serum marker for clinical severity in patients with scleroderma? *Br J Dermatol* 139:1020–1025, 1998

Hyland J, Ala-Kokko L, Royce P, Steinmann B, Kivirikko K, Myllylä R: A homozygous stop codon in the lysyl hydroxylase gene in two siblings with Ehlers–Danlos syndrome type VI. *Nature Genet* 2:228–231, 1992

Ishikawa O, Kondo A, Miyachi Y: Mature type of skin collagen cross-link, histidinohydroxylysinonorleucine, is significantly increased in the skin of systemic sclerosis. *Arthritis Rheum* 41:376–377, 1998

Kerscher M, Volkenandt M, Meurer M, Lehmann P, Plewig G, Roecken M: Treatment of localized scleroderma with PUVA bath chemotherapy. *Lancet* 343:1233, 1994

Knott L, Bailey AJ: Collagen cross-links in mineralizing tissues: a review of their chemistry, function and clinical relevance. *Bone* 22:181–187, 1998

Last JA, King TE Jr, Nerlich AG, Reiser KM: Collagen cross-linking in adult patients with acute and chronic fibrotic lung disease. *Am Rev Respir Dis* 141:307–313, 1990

Meddah B, Kamel S, Giroud C, Brazier M: Effects of ultraviolet light on free and peptide-bound pyridinoline and deoxypyridinoline cross-links. Protective effect of acid pH against photolytic degradation. *J Photochem Photobiol B Biol* 54:168–174, 2000

Moriguchi T, Fujimoto D: Crosslink of collagen in hypertrophic scar. *J Invest Dermatol* 72:43–145, 1979

Oxlund H, Barckman M, Ortoft G, Andreassen TT: Reduced concentrations of collagen cross-links are associated with reduced strength of bone. *Bone* 17:365S–371S, 1995

Petersen MJ, Hansen C, Craig S: Ultraviolet A irradiation stimulates collagenase production in cultured human fibroblasts. *J Invest Dermatol* 99:440–444, 1992

Reiser K, McCormick RJ, Rucker RB: Enzymatic and nonenzymatic cross-linking of collagen and elastin. *FASEB J* 6:2439–2449, 1992

Ricard-Blum S, Esterre P, Grimaud JA: Collagen cross-linking by pyridinoline occurs in non-reversible skin fibrosis. *Cell Mol Biol* 39:723–727, 1993

Sakura S, Fujimoto D, Sakamoto K, Mizuno A, Motegi K: Photolysis of pyridinoline, a cross-linking amino acid of collagen, by ultraviolet light. *Can J Biochem* 60:525–529, 1982

Sassi ML, Jukkola A, Riekki R, Höyhtyä M, Risteli L, Oikarinen A, Risteli J: Type I collagen turnover and cross-linking are increased in irradiated skin of breast cancer patients. *Radiother Oncol*, in press

Scharffetter K, Wlaschek M, Hogg A, *et al*: UVA irradiation induces collagenase in human dermal fibroblasts *in vitro* and *in vivo*. *Arch Dermatol Res* 283:506–511, 1991

Scharffetter-Kochanek K, Goldermann R, Lehmann P, Holzle E, Goerz G: PUVA therapy in disabling pansclerotic morphoea of children [letter]. *Br J Dermatol* 132:830–831, 1995

Scharffetter-Kochanek K, Brenneisen P, Wenk J, *et al*: Photoaging of the skin from phenotype to mechanisms. *Exp Gerontol* 35:307–316, 2000

Sims TJ, Avery NC, Bailey AJ: Quantitative determination of collagen crosslinks. *Meth Mol Biol* 139:11–26, 2000

Stege H, Berneburg M, Humke S, *et al*: High-dose UVA1 radiation therapy for localized scleroderma. *J Am Acad Dermatol* 36:938–944, 1997

Uzawa K, Marshall MK, Katz EP, Tanzawa H, Yeowell HN, Yamauchi M: Altered posttranslational modifications of collagen in keloid. *Biochem Biophys Res Com* 249:652–655, 1998

Vater CA, Harris ED Jr, Siegel RC: Native cross-links in collagen fibrils induce resistance to human synovial collagenase. *Biochem J* 181:639–645, 1979

Yamauchi M, London RE, Guenat C, Hashimoto F, Mechanic GL: Structure and formation of a stable histidine-based trifunctional cross-link in skin collagen. *J Biol Chem* 262:11428–11434, 1987

Yamauchi M, Prisayanh P, Haque Z, Woodley DT: Collagen cross-linking in sun-exposed and unexposed sites of aged human skin. *J Invest Deramtol* 97:938–941, 1991

Yamauchi M, Chandler GS, Tanzawa H, Katz EP: Cross-linking and the molecular packing of corneal collagen. *Biochem Biophys Res Com* 219:311–315, 1996